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Origin, Biogenesis, and Activity of Plant MicroRNAs

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MicroRNAs (miRNAs) are key posttranscriptional regulators of eukaryotic gene expression. Plants use highly conserved as well as more recently evolved, species-specific miRNAs to control a vast array of biological processes. This Review discusses current advances in our understanding of the origin, biogenesis, and mode of action of plant miRNAs and draws comparisons with their metazoan counterparts.

MicroRNAs (miRNAs) are fundamental, sequence-specific regulatory elements of eukaryotic genomes. In plants, these molecules are found amid a maelstrom of heterogeneous, 19–24 nucleotide (nt)-long RNA species called short-interfering RNAs (siRNAs) that mediate endogenous gene silencing at both the transcriptional and posttranscriptional levels. Recent biochemical and genetic investigations of their possible origin, biogenesis, and modes of action indicate that plant miRNAs share many similarities with their animal counterparts (see Review by R.W. Carthew and E.J. Sontheimer on page 642 of this issue). These studies have also uncovered several regulatory layers that control the processing and activity of plant miRNAs and may well be of relevance in metazoans. They further indicate that the distinction between microRNAs and siRNAs in plants is being progressively blurred, as both types of molecule seem intimately linked in terms of their origins and modes of operation through related or identical effector proteins. Collectively, these findings have important implications for our current understanding of plant miRNA-mediated regulation at the cellular and organismal levels. This Review summarizes our knowledge pertaining to the possible origins of the genes encoding plant miRNAs (*MIR* genes), the mechanisms of their biogenesis and action, and the variety of processes that modulate these mechanisms.

The Small RNA Multitude in Plants

Small RNAs (sRNAs), many of which are involved in mediating the silencing of gene expression, abound in plants. The plant RNA-silencing phenomena share four consensus biochemical steps: (1) induction by double-stranded RNA (dsRNA), (2) dsRNA processing into 18–25 nucleotide (nt)-long sRNAs, (3) 3′-O-methylation of sRNA, and (4) sRNA incorporation into effector complexes that associate with partially or fully complementary target RNA or DNA (reviewed in Chapman and Carrington, 2007). Double-stranded RNA might derive directly from virus replication, transcription of inverted-repeat sequences, or convergent transcription. The generation of dsRNA may also be "genetically programmed" at endogenous loci that produce transcripts with internal stem-loop structures. Alternatively, dsRNA may be synthesized by one of six RNA-dependent RNA polymerases (RDR1–6) that copy single-stranded RNA (ssRNA). RDR templates include mRNAs with aberrant features or transcripts produced by a putative plant-specific RNA polymerase IV (Pol IV) whose subunit NRPD1a is found at certain methylated genomic loci. In the plant *Arabidopsis thaliana*, the dsRNA is processed into specifically sized sRNA duplexes by one of four Dicer-like (DCL1-4) proteins. DCL1 mainly produces 18–21 nt-long sRNA. In contrast, the products of DCL2, DCL3, and DCL4 are 22 nt, 24 nt, and 21 nt long, respectively.

Upon dicing, sRNA duplexes are either retained in the nucleus for chromatin-level activities or exported to the cytoplasm for posttranscriptional gene silencing (PTGS). A selected sRNA strand incorporates into one or several RNA-induced silencing complexes (RISCs) that scan the cell for complementary nucleic acids to execute their function. Plant sRNA-directed RISC activities include (1) RNA endonucleolytic cleavage or "slicing" at the center of sRNA-target hybrids, (2) repression of translation through unknown mechanisms, and (3) DNA cytosine and/or histone methylation, in the latter case with the assistance of Pol IV subunit b (NRPD1b). Eukaryotic RISCs invariably contain one member of the ARGONAUTE (AGO) protein family, which have an sRNA-binding PAZ domain and a PIWI domain with catalytic residues conferring endonucleolytic activity to those RISCs programmed to slice RNAs. Among the ten predicted *Arabidopsis* family members (AGO1–10), roles for AGO1, AGO4, AGO6, and AGO7 in sRNA-directed silencing have been established, and "slicer" activity has been demonstrated for both AGO1 and AGO4 (reviewed in Vaucheret, 2008). High-throughput cloning and sequencing show that the plant sRNA repertoire is vastly dominated by an ocean of siRNAs acting mostly at the chromatin level and mapping to transposon loci and DNA repeats. These heterochromatin-associated 24 nt siRNAs are diced by DCL3 or its surrogate DCL2 and incorporated into AGO4 or AGO6 to guide cytosine methylation in all sequence contexts, a landmark of RNA-directed DNA methylation (RdDM). They are often referred to as *cis*-acting siRNAs because they affect the genomic loci that produce them, often resulting in their transcriptional gene silencing (TGS).

MicroRNAs amid the Small RNAs

The second most abundant class of plant sRNAs are miRNAs. These small RNAs are most easily detected in *rdr2* or *dcl3*

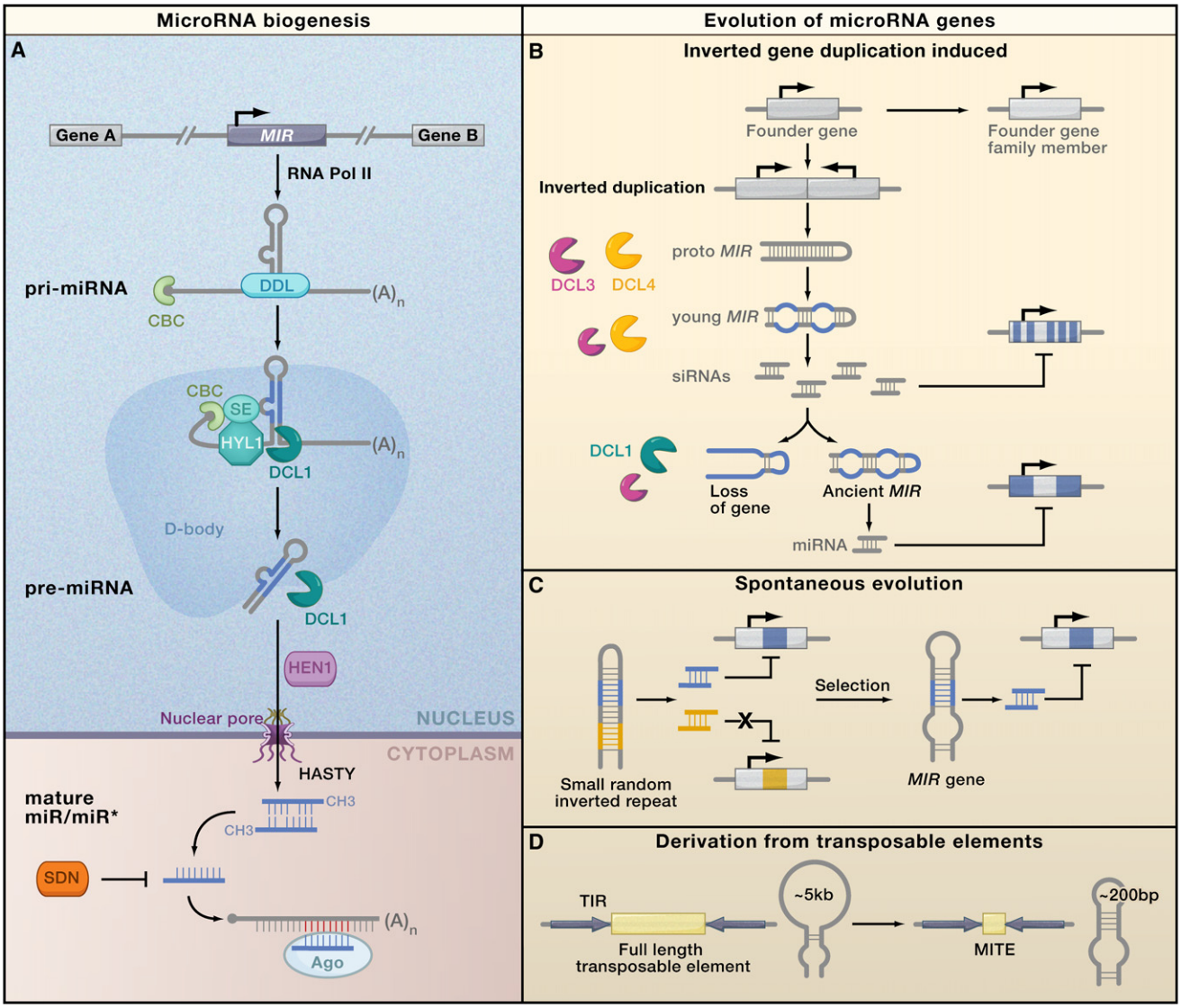

Figure 1. Origins and Biogenesis of Plant miRNAs

(A) Molecular pathway for the processing and stability of conserved plant microRNAs (miRNAs). Plant pri-miRNAs are mostly transcribed by RNA polymerase II (Pol II) from regions located between protein-coding genes. The RNA-binding protein DAWDLE (DDL) presumably stabilizes pri-miRNAs for their conversion in nuclear processing centers called D-bodies to stem-loop pre-miRNAs. This reaction entails the concerted action and physical interaction of the C2H2-zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), Dicer-like 1 (DCL1), and nuclear cap-binding complex (CBC). Pre-miRNAs, or mature miRNAs produced by DCL1, are then exported to the cytoplasm possibly through the action of the plant exportin 5 ortholog HASTY and other unknown factors. Mature RNA duplexes excised from pre-miRNAs (miRNA/miRNA*, where miRNA is the guide strand and miRNA* is the degraded strand) are methylated by HEN1, a reaction that protects them from being degraded by the SMALL RNA DEGRADING NUCLEASE (SDN) class of exonucleases. The guide miRNA strand is then incorporated into AGO proteins to carry out the silencing reactions.

(B) Evolution of a young miRNA-encoding (*MIR*) gene through an inverted duplication event. A double-stranded RNA with perfect base pairing is initially generated and processed into siRNA populations by DCL3 and DCL4 (proto-*MIR*). The stem-loop region progressively acquires mutations (blue) that contribute to shortening and to the gain of bulges in the dsRNA structure. The resulting young *MIR* is now prevalently diced by DCL4, producing *trans*-acting small RNA molecules. One of these RNAs undergoes preferential selection through the concerted evolution of a target gene initially spawned by proliferation of the founder gene. Lack of selection would result in genetic drift such that the young *MIR* gene would eventually be lost. Continued selection would shape the young *MIR* gene into an ancient *MIR* gene in which the size and various bulges in the stem loop are now compatible with the processing by DCL1 to a single miRNA molecule. Concerted evolution of the target gene would contribute to selection of a discrete miRNA target site (in the coding region, for example).

(C) Spontaneous evolution of *MIR* genes from small, random inverted repeats scattered along plant genomes. siRNAs generated from such repeats would fortuitously match complementary regions found in potential target genes. Selection of the targeting event under appropriate circumstances would then contribute to isolation of a single miRNA molecule within the stem-loop structure.

knockout backgrounds that prevent the accumulation of the abundant *cis*-acting siRNAs. Plant miRNAs are part of a near-ubiquitous class of 20–24 nt RNA molecules that regulate eukaryotic gene expression posttranscriptionally. miRNAs use base pairing to guide RISCs to specific messages bearing fully or partly complementary sequences (Figure 1A). Repression of the target transcript by miRNAs may occur through translational inhibition, accelerated exonucleolytic mRNA decay, or slicing within miRNA-mRNA base pairing (Eulalio et al., 2008). Most characterized eukaryotic *MIR* genes are RNA polymerase II (Pol II) transcription units that yield a primary miRNA transcript called a pri-miRNA (Lee et al., 2004). The pri-miRNA typically forms an imperfect fold-back structure, which is processed into a stem-loop precursor (pre-miRNA, Figure 1A) and further excised as an RNA duplex (Kim, 2005).

In animals, the duplex strand with the weakest 5′end base pairing is then selected as the mature miRNA and loaded into an AGO protein. The remaining strand, called miRNA*, is degraded (Tomari et al., 2004). Plant and metazoan miRNAs were initially identified as a result of their roles in developmental patterning. miRNA-deficient mutants in both kingdoms exhibit severe developmental defects because they fail to acquire appropriate cell identities during embryonic and post-embryonic growth (Bernstein et al., 2003; Jacobsen et al., 1999). A number of miRNA target transcripts were indeed identified as transcription factors or general growth regulators crucial for cell fate determination (Reinhart et al., 2000; Rhoades et al., 2002; Wightman et al., 1993). This initially led to the proposal that miRNAs might have evolved as a consequence of multicellularization, but it was later discovered that single-cell organisms such as the green algae *Chlamydomonas reinhardtii* also produce miRNAs (Molnar et al., 2007; Zhao et al., 2007). Moreover, a wealth of studies further established that, beyond their key roles in development (reviewed in Flynt and Lai, 2008; Garcia, 2008), hundreds of eukaryotic miRNAs seem to regulate a vast array of other biological functions including hormonal control, immune responses, and adaptation to a variety of biotic and nonbiotic stresses (reviewed in Pedersen and David, 2008; Sunkar et al., 2007; Voinnet, 2008a).

Despite their resemblance, plant and metazoan miRNAs have been mostly investigated as largely distinct entities, mainly because of the seemingly different ways in which they appear to regulate their targets upon loading into AGO proteins. Typically, metazoan miRNAs are thought to regulate transcripts through imperfect complementarity to multiple sites found in 3′ untranslated regions (UTRs). In animals, imperfect miRNA-mRNA hybrids with central bulges (nucleotides 9–12) generally account for regulation that occurs mostly through translational inhibition and only rarely by slicing. Owing to these relaxed base-pairing requirements, individual metazoan miRNAs may have dozens of target transcripts. Plant miRNAs, by contrast, are thought to largely regulate transcripts by single, highly complementary target sites in coding regions. Thus, a large number of plant miRNAs may function through slicing, which is thought to constitute an efficient means of "mRNA clearance." Consequently, plant miRNAs are predicted to have only a limited number of mRNA targets.

Plant *MIR* Genes: Young and Old

Unlike most metazoan *MIR* genes, which are mainly found within introns or exons, most plant *MIR* genes are intergenic. Their fold-back structure and length are also more variable. Animal *MIR* genes are often genomically clustered and cotranscribed as polycistronic RNAs (Kim, 2005). In contrast, plant *MIR* genes are rarely arranged in tandem, although clustering seems not uncommon in some plants such as soybean (Zhang et al., 2008a). Among the near-thousand plant *MIR* genes deposited in the miRbase registry (http://microrna.sanger.ac.uk/sequences), ~100 families of related miRNAs form an evolutionary fluid set of molecules in *Arabidopsis*. At one extreme, some miRNA families are conserved in moss, indicating their very ancient origin. These include *miR-156*, *miR-160*, *miR-319*, and *miR-390*, all of which regulate ancestral transcription factors that specify basic meristem functions, organ polarity and separation, cell division, or hormonal control (reviewed in Garcia, 2008). Notably, none of the miRNAs identified in the single-cell algae *Chlamydomonas* seem conserved in multicellular plants (Molnar et al., 2007). Other miRNA families evolved after the split between land plants and mosses but before the monocot/dicot divergence. Twenty-one such miRNA families seem universal among angiosperms (reviewed in Axtell and Bowman, 2008). Many conserved plant *MIR* genes arose through extensive genome duplications and rearrangements and thus often have multiple loci. Also, these genes are usually highly expressed, a feature which, until the advent of deep-sequencing technologies, hindered the identification of a second large class of low-to-moderately abundant "younger" miRNAs in plants.

Some of these evolutionarily recent miRNAs—the other extreme in plant *MIR* gene evolution—were detected early on and found to be typically represented by single-copy genes not conserved in phylogenies (Zhang et al., 2006). Their predicted targets in *Arabidopsis* (many await experimental validation) include a much broader range of proteins, encompassing virtually all aspects of plant biology, than those regulated by conserved miRNAs. Several exhaustive studies have shown that *Arabidopsis* nonconserved miRNAs already largely outnumber their conserved counterparts, the identification of which seems to have reached a plateau (Fahlgren et al., 2007; Rajagopalan et al., 2006; Zhang et al., 2006). The limited overlap between the results of these studies further suggests that more nonconserved miRNAs will be discovered, owing to induced or restricted expression, such as that dependent upon stresses or cell-type specificity. Related analyses in bryophytes and lycophytes also showed a similar diversity of lineage-specific miRNAs (Talmor-Neiman et al., 2006), unraveling a probable universal feature of land plants.

Analysis of a particular class of such young miRNAs in *Arabidopsis* suggests one potential origin of plant *MIR* genes

(D) *MIR* gene evolution through generation of miniature inverted-repeat transposable elements (MITEs). A full-length DNA-type transposable element (TE) with terminal inverted repeats (TIRs) flanking a long open reading frame is shown. The snap-back secondary structure potentially resulting from annealing of the TIRs is incompatible with small RNA processing. Transposable elements may give rise to MITEs, nonautonomous derivatives of full-length DNA-type elements containing TIRs and a small internal region. The small imperfect hairpin resulting from MITE transcription resembles a pre-miRNA precursor.

(Figure 1B). These miRNAs have precursors that show extensive sequence similarity to their target genes, suggesting that inverted gene duplication events generated them (Allen et al., 2004; Fahlgren et al., 2007). Such inverted loci would initially produce heterogeneous siRNA populations resembling those processed by DCL4 and DCL3 from perfect, transgenic RNA interference (RNAi) hairpins (Dunoyer et al., 2007; Figure 1B). Acquisition of DCL1 dependence and subsequent production of discrete small RNA species would then require accumulation of drift mutations that cause fold-back mispairing. Without selection, these proto-*MIR*s would drift further, eventually becoming inert. Alternatively, target gene regulation might become advantageous under conditions of stress or reproductive isolation, thereby selecting the miRNA within the surrounding fold-back sequence to produce a young *MIR* gene (Figure 1B). Continued selection would enforce further drift of fold-back arm sequences surrounding the miRNA/miRNA*, ultimately generating an old *MIR* gene unrelated to the parental locus (Figure 1B; Allen et al., 2004; Fahlgren et al., 2007). This gene-duplication model is consistent with young *MIR* genes having low levels of expression, which may be potentially required to reduce the off-targeting costs of early-phase siRNA-like populations. This model is also substantiated by recent in-depth analyses confirming a progressive shift in DCL usage (DCL4 to DCL1) from young to old *MIR* genes in *Arabidopsis* (Figure 1B) (Rajagopalan et al., 2006; Vazquez et al., 2008). However, this model postulates that *MIR* gene evolution is mostly constrained by interactions between mature miRNA fragments and target exons. This is inconsistent with an analysis of *Arabidopsis*-specific *MIR-824* showing that nonrandom evolution of thermo-resistant structures within the pre-miRNA fold-back impacts processing efficacy (de Meaux et al., 2008). Thus, there are likely additional ways in which to evolve young *MIR* genes, two of which were suggested in recent genomic surveys.

The first possible model of evolution, termed "spontaneous evolution," stems from the high density of small-to-medium sized fold-back sequences scattered throughout the *Arabidopsis* genome (Figure 1C). Following the capture of transcriptional regulatory sequences, some of these random fold-backs could occasionally give rise to new *MIR* genes. Stabilization through coevolution with targets initially found by chance could then lead to the fixation of these genes in the genome (de Felippes et al., 2008). The second model (Figure 1D) relies on the recent observation that DNA-type nonautonomous elements known as miniature inverted-repeat transposable elements (MITEs) readily fold into imperfect stem loops typical of miRNA precursors (Piriyapongsa and Jordan, 2008). Accordingly, several putative young miRNAs in rice and, to a lesser extent, in *Arabidopsis* map genomically to MITEs and other transposable element loci, as is commonly seen with animal *MIR* genes. However, 24 nt-long *cis*-acting, DCL3-dependent siRNAs involved in taming transcription of transposable elements are also abundant at those loci. Thus it remains debatable whether these small RNAs are indeed bona fide miRNAs and not siRNAs. Nonetheless, a transposable element-based origin of some plant miRNA may explain why many conserved *MIR* genes in *Arabidopsis* are processed as both 21 nt and 24 nt species by DCL1 and DCL3, respectively (Figure 1B; Dunoyer et al., 2004; Vazquez et al., 2008).

Irrespective of their origin, young *MIR* genes are expected to emerge and dissipate over short evolutionary timescales, a hypothesis now testable by deep-sequence analysis of small RNAs isolated from *Arabidopsis* relatives and from *Arabidopsis* ecotypes with drastically different habitats. Such a study performed in different species of the fly genus *Drosophila* recently unraveled a large class of low-expressed, nonconserved miRNAs, of which only 4% were eventually maintained by natural selection. Furthermore, only a small fraction of those genes were retained as regulatory elements in the long term, uncovering, indeed, high rates of birth and death for fly *MIR* genes (Lu et al., 2008). The young fly miRNAs seemed not to originate by inverted duplication but rather from small fold-back sequences akin to those inferred from the spontaneous evolution hypothesis in *Arabidopsis*.

The proposed fluidity of nonconserved plant *MIR* genes does not imply that ancient miRNAs are necessarily evolutionary rigid. Many highly conserved miRNAs respond to environmental stresses and aid in plant adaptation. Moreover, point mutations in their genes are common across plant species, where they may confer adaptive advantages. Indeed, several flooding-responsive *MIR* genes of maize display single-nucleotide polymorphisms in comparison to their orthologs in *Arabidopsis* or pine. These polymorphisms extend the predicted set of maize targets to genes involved in carbohydrate/energy metabolism and in the elimination of toxic compounds produced during water submergence-induced anaerobia (Zhang et al., 2008b). Studies of miR-319a orthologs have also indicated that conserved *MIR* genes can evolve through changes in pre-miRNA structure that cause species-dependent processing differences or through the divergence of *cis*-regulatory elements that affect their transcriptional patterns (Palatnik et al., 2007; Warthmann et al., 2008).

Plant miRNA Processing and Stability

RNA Pol II produces capped and polyadenylated pri-miRNAs in plants and animals. However, both the pri-to-pre-miRNA conversion and mature miRNA processing are orchestrated by DCL1 in plants, which lack the Drosha-like enzyme of metazoans (Figure 1A; Kurihara et al., 2006). pri-to-pre-miRNA conversion also necessitates the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1) and the C2H2-zinc finger protein SERRATE (SE), which interact with DCL1 in nuclear processing centers called D-bodies or SmD3/SmB-bodies (Fang and Spector, 2007; Kurihara et al., 2006). Whereas null *DCL1* or *SE* alleles are embryonic-lethal, alleles of *se* that weakly perturb SERRATE function give rise to phenotypes that resemble those caused by mutations in *ABH1/CBP80* and *CBP20,* which encode subunits of the nuclear cap-binding complex (CBC) (Gregory et al., 2008; Laubinger et al., 2008). Like *se* mutants, *abh1/cbp80* and *cbp20* mutants accumulate more pri-miRNAs and less mature miRNAs. Weak *se* mutants exhibit the general mRNA splicing defects typical of *abh1/cbp80* and *cbp20* mutants. Thus, dual roles in splicing and miRNA processing distinguish SE and CBC from the specialized pri-to-pre-miRNA processing factors, DCL1 and HYL1 (Figure 1A). Similar roles for animal CBC are probable, as a CBC component seems to be required for miRNA function in the nematode *Caenorhabditis*

elegans (Parry et al., 2007). miRNA accumulation is also low in the pleiotropic *Arabidopsis dawdle (ddl)* mutant, but unlike in *se* or *hyl1* mutants, pri-miRNAs accumulate poorly in *ddl* mutants despite unaltered *MIR* transcription (Yu et al., 2008). The *DDL*-encoded nuclear RNA-binding protein thus likely stabilizes pri-miRNAs, consistent with it interacting directly with DCL1 (Figure 1A; Yu et al., 2008). DDL functions probably extend beyond miRNA biogenesis, as the mutant shows stronger developmental abnormalities than *dcl1* and lacks several DCL3-dependent siRNA species. DDL also seems substrate nonspecific, as it binds equally to in vitro transcribed mRNAs and pri-miRNAs (Yu et al., 2008). The related human SNIP1 (Smad nuclear-interacting protein 1) is likely orthologous to DDL because it interacts with Drosha but not with human Dicer, and its knockdown compromises miRNA accumulation in HeLa cells (Yu et al., 2008).

Mature miRNA duplexes are stabilized by the S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1), which methylates all plant-silencing small RNAs (Figure 1A). Methyl groups deposited on the 3′ terminal nucleotides of each strand prevent their uridylation and subsequent degradation, explaining the shared developmental defects of *hen1* and hypomorphic *dcl1* mutants (Li et al., 2005; Yang et al., 2006). The HEN1 ortholog (dmHEN1) in the fly *Drosophila melanogaster* methylates single-stranded siRNAs involved in experimental RNAi and endogenous regulations (Horwich et al., 2007). PIWI-interacting RNAs (piRNAs) thought to tame transposons in *Drosophila* and mouse germlines also are methylated by dmHEN1 and mmHEN1, respectively, but fly and mammalian miRNAs seem to not undergo this modification (reviewed in Aravin et al., 2007). The recent isolation of *Arabidopsis* exonucleases that degrade miRNA single strands in vitro further underscores the importance of miRNA stability control in plants (Ramachandran and Chen, 2008). Simultaneous knockdown of three members of this *SMALL RNA DEGRADING NUCLEASE* (SDN; Figure 1A) gene family elevates miRNA levels and causes developmental defects in *Arabidopsis*. Eukaryotic SDN proteins are highly conserved, so their animal homologs might similarly metabolize silencing small RNAs, including miRNAs. Plant SDNs differ from Enhanced RNAi-1 (Eri-1) nucleases originally identified in *C. elegans* (Kennedy et al., 2004), of which there are also six putative homologs in *Arabidopsis* (Ramachandran and Chen, 2008). Eri-1-like proteins specifically degrade siRNA duplexes with two nucleotide long 3′ overhangs.

HASTY, the plant homolog of exportin-5, is required for miRNA function coincident with or following miRNA biogenesis (Park et al., 2005) (Figure 1A). The role of exportin-5 is not as clear as in animals, where pre-miRNAs are experimentally verified cargoes. *hasty* mutants show decreased accumulation of only some miRNAs, in both nuclear and cytoplasmic fractions (Park et al., 2005), suggesting the existence of HASTY-independent miRNA export systems. The exact exported form of plant miRNAs is unclear, as locations of miRNA/miRNA* strand separation and miRISC loading are elusive in plants, as are the sites of miRNA methylation and SDN-mediated degradation. Nonetheless, these analyses highlight a surprising degree of overall similarity between the mechanisms of plant and metazoan small RNA biogenesis and stability.

Plant miRNA Modes of Operation

It was observed early on that evolutionarily conserved miRNAs have readily identifiable target sites in mRNA open reading frames (ORFs) in *Arabidopsis*, to which they are extensively complementary (Rhoades et al., 2002). Extensive pairing was found to encompass nucleotides 9–11, suggesting a "slicing" mode of action for these molecules, resembling siRNA-directed silencing. Cleaved RNA fragments mapping to central regions of predicted hybrids were indeed retrieved in experiments using the technique of rapid amplification of 5′ complementary DNA ends (5′ RACE); stable 3′-cleavage fragments diagnostic of slicing were also routinely detected by northern analyses (Dunoyer et al., 2004; Llave et al., 2002; Souret et al., 2004). Until 2007, antibodies against the gene products of miRNA targets remained little used in studies of plant miRNA-target interactions. In those studies where protein production was measured, a discrepancy was observed between the extent of miRNA-directed transcript degradation and the loss of protein accumulation (Aukerman and Sakai, 2003; Bari et al., 2006; Chen, 2003; Gandikota et al., 2007), suggesting that plant miRNAs may also inhibit protein production from their targets.

These important observations were indeed confirmed and broadened by the results of a forward genetics screen for *miRNA-action deficient* (mad) mutants in *Arabidopsis* (Brodersen et al., 2008). Characterization of the identified *mad* mutants (*mad1–6*) showed that the action of most plant miRNA commonly entails a combination of target degradation (compromised in *mad* class-II mutants) and translational repression (compromised in *mad* class-III mutants), which presumably affects the pool of transcripts remaining after cleavage (Figure 2A, center). Importantly, neither the position (ORF, 5′ or 3′UTR) nor the degree of pairing at the miRNA target sites appeared to be predictive of the prevalence of one process over the other. This supports the previous suggestion that transcript cleavage and translational inhibition have similar sequence requirements as in animals and also indicates that the later-acting mode of repression by translational inhibition is not simply a "back-up" system for slicing (Brodersen et al., 2008; Palatnik et al., 2007). Widespread translational repression by plant miRNAs is consistent with the substantial levels of full-length target mRNAs that often remain detectable by northern blot analysis following miRNA action (Dunoyer et al., 2004; Llave et al., 2002; Souret et al., 2004). Furthermore, 5′ RACE, the technique predominantly used to validate miRNA targets, is a qualitative procedure and does not indicate the extent of slicing. Likewise, overexpression of endogenous or artificial miRNA phenocopies the effects of knockout mutations despite appreciable accumulation of target mRNAs (Bari et al., 2006; Schwab et al., 2006). Furthermore, central nucleotide mismatches in miRNA-target pairs that are predicted (and occasionally confirmed) to alter slicing rates had modest phenotypic consequences in several ectopic expression experiments (see for example, Mallory et al., 2004).

Most miRNAs are loaded into and probably affected by AGO1, one of ten *Arabidopsis* Argonaute paralogs (reviewed in Vaucheret, 2008). AGO1 slices miRNA targets (Baumberger and Baulcombe, 2005) and also apparently represses their translation, as evidenced with hypomorphic *ago1–27* mutants (Brodersen et al., 2008). These mutants exhibit near-normal levels

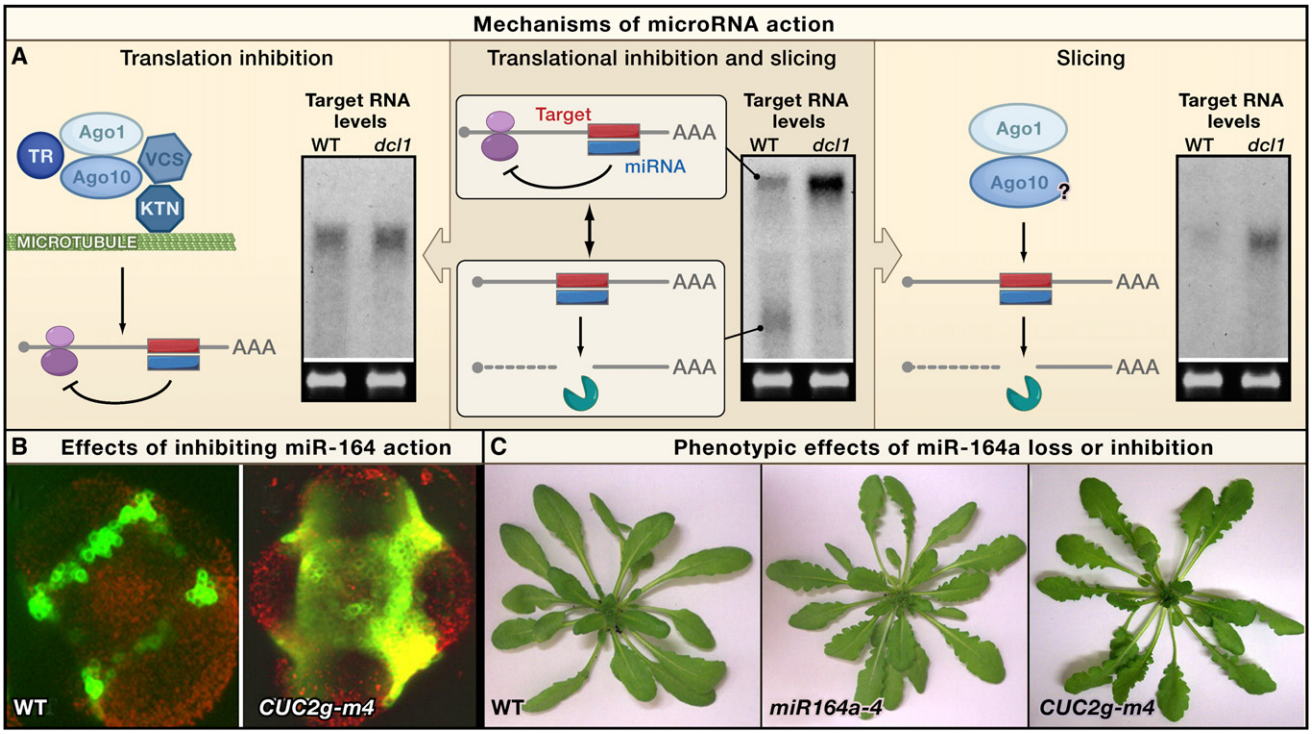

Figure 2. Modes of miRNA Action

(A) (Center) Dual modes of miRNA action entail a combination of slicing and translational repression. Shown is the typical molecular output of regulations affecting most plant miRNAs targets, as detected by a northern blot. The right lane on the blot contains RNA extracted from the *Arabidopsis* miRNA-deficient mutant *dcl1*, used as a negative control. The left lane contains RNA from wild-type (WT) plants. The accumulation of a fast-migrating 3′ cleavage mRNA fragment diagnoses the occurrence of slicing, but high levels of full-length mRNAs remain and may be subject to translational repression, as suggested by the genetic analysis (Brodersen et al., 2008). (Right) One possible extreme case where the miRNA entirely regulates its target at the mRNA level. (Left) One possible extreme case where the miRNA entirely regulates its target at the protein level, pending the presence of hypothetical translational repressors (TR) that might prevent the slicing activity of miRNA-loaded ARGONAUTE 1 (AGO1) and AGO10. Translation inhibition also specifically requires the microtubule (MT) severing activity of KATANIN (KTN) and the action of the *Ge-1* ortholog VARICOSE (VCS). Note that the role of AGO10 in miRNA-guided slicing is currently unclear, as is the nature of the factors required for AGO-mediated slicing.

(B) (Left) In *Arabidopsis*, *miR-164* normally constrains the expression domain of its target gene *CUP-SHAPED COTYLEDON* (*CUC2*). CUC2 protein is visualized here as a green fluorescent protein fusion. (Right) Plants expressing an miRNA-resistant form of CUC2 (*CUC2g-m4*) show aberrant, enlarged domains of CUC2 expression.

(C) Relative to wild-type plants (left), the phenotype of plants lacking individual *miR-164* paralogs (center) suggests that the isoform *miR-164a* predominantly contributes to preventing the developmental anomalies (leaf serration) caused by expression of the *CUC2g-m4* construct (right). Only very rarely are such genetic studies conducted to validate the phenotypes of miRNA-resistant transgenic lines in *Arabidopsis*. Images from Nikovics et al. (2006), http://www.plantcell.org, copyright American Society of Plant Biologists.

of miRNA target transcripts yet accumulate disproportionately high levels of protein from these transcripts. They also display overlapping developmental defects with *dcl1* mutants. Presumably, the point mutation in *ago1–27* (Morel et al., 2002) uncouples slicing activity (which remains intact) from translational repression by disrupting interactions between AGO1 and other proteins specifically required for translation repression (Figure 2A, left). Accordingly, *Arabidopsis* AGO1 protein complexes (J. Azevedo and O.V., unpublished) prevalently contain factors that are homologous to hsAGO2-interacting proteins required for miRNA-mediated translational repression in HeLa cells (Beitzinger et al., 2007). A mutation in *AGO10/PNH/ZLL*, the closest *AGO1* paralog, causes developmental defects typical of *ago1* mutants and also suppresses miRNA-mediated translational effects (Brodersen et al., 2008; Lynn et al., 1999; Moussian et al., 1998). However, only some (and not necessarily the same) miRNA-target regulations seem compromised in *ago1* ands *ago10* mutants (Brodersen et al., 2008). Because AGO1 and AGO10 expression domains overlap only partially, and because *ago10* mutants show apical meristem defects not seen in *ago1* (Lynn et al., 1999; Moussian et al., 1998), each protein might recruit selected subsets of miRNAs in specific tissues or at specific developmental stages. Likewise, translational repressors coupled to AGO1 or AGO10 action might be available only in specific cell types, such that slicing and translational repression might be, at times, spatially or temporally separated (Figure 2A, left).

Other factors required specifically for translational repression in *Arabidopsis* include the *MAD5*-encoded microtubule-severing enzyme KATANIN (Brodersen et al., 2008). Similar requirements for tubulins in *C. elegans* suggest that cytoskeleton dynamics is a universal but underappreciated element of miRNA action (Parry et al., 2007). The P-body component VARICOSE (VCS)

(Goeres et al., 2007) is also integral to translational repression by *Arabidopsis* miRNAs (Brodersen et al., 2008). VCS is orthologous to Ge-1, required for decapping of miRNA targets in *Drosophila* (Eulalio et al., 2007). Thus, mRNA decay might be coupled to the translational repression of at least some plant miRNA targets (Figure 2A, left), as is the case in animals.

Plant miRNA-Directed Regulation

The default state of plant miRNAs is, therefore, to bring their targets under translational as well as RNA stability control, with the two layers of regulation not necessarily coinciding spatially or temporally. At the organism or organ level, this default regulatory state would be diagnosed by northern blot as two populations of cleaved and uncleaved RNA transcripts (Figure 2A, middle). This is indeed the stereotypical molecular phenotype observed for most plant miRNA targets (Dunoyer et al., 2004; Llave et al., 2002; Souret et al., 2004). From these data, the miRNA-mediated regulation could be deconvoluted into two theoretically separable states. In the first theoretical state (Figure 2A, right), miRNAs would predominantly operate through transcript cleavage. This mode of action would be ideally suited to produce irreversible switches that are required, for instance, to establish permanent cell fates during embryogenesis or in adult stem cell niches of plant apexes (meristems). The dramatic developmental consequences of perturbing slicing, as opposed to translational repression (comparing the *mad* mutant classes or analyzing slicer-deficient *AGO1* alleles), probably reflect the importance of this type of regulation in plant development (Baumberger and Baulcombe, 2005; Brodersen et al., 2008). Nonetheless, target "tuning," as opposed to switching on or off, is also a possible outcome of slicing. For example, the *Arabidopsis miR-164*a mutant shows stochastic extension of the expression domains of its floral target, CUP-SHAPED COTYLEDON (CUC), strongly suggesting that *miR-164* has thresholding functions that normally delineate the regions of CUC activity (Nikovics et al., 2006; Sieber et al., 2007; Figures 2B and 2C).

In the second theoretical regulatory state (Figure 2A, left panel), miRNAs would mainly repress target protein production in a reversible manner, as inferred from studies in metazoans (Bhattacharyya et al., 2006; Schratt et al., 2006). In principle, this type of regulation could regulate cell fate-promoting decisions, as with slicing. Reversible translational repression would also be well suited to coordinating and resetting stress-responsive gene expression, an emerging function of many plant miRNAs (reviewed in Sunkar et al., 2007; Voinnet, 2008a). For instance, both *miR-399* (induced by phosphate starvation) and *miR-393* (induced during infection by the bacterium *Pseudomonas syringae*) suppress negative regulators of phosphate mobilization and plant basal defense, respectively, by acting predominantly at the protein production level (Bari et al., 2006; Fujii et al., 2005; Navarro et al., 2006). Reversibility, in both cases, would ensure that translation of negative regulators resumes immediately after the stress is gone, thereby reducing the fitness costs of prolonged stress response activation.

Ultimately, miRNA regulation purely by cleavage or purely by translational inhibition is probably rare, should it exist. A continuum of plant miRNA action involving a blend of both mechanisms would have the advantage of adding significant flexibility to miRNA functions, depending on the presence or absence of AGO-interacting translational repressor proteins in particular tissues (Figure 2A). At the systems level, this might add versatility to the prototypical regulatory circuits orchestrated by plant miRNAs, which resemble those found in metazoans (Flynt and Lai, 2008). These circuits include, but are not limited to, spatial restriction, temporal regulation, mutual exclusion of the miRNA and target gene, as well as the dampening of target gene expression (Figure 3). The relative levels of accumulation of an miRNA and its targets could conceivably also influence the type of circuit involved.

Modulating Plant miRNA Expression, Processing, and Action

The expression and activity of plant miRNA can be regulated in many ways. These include transcriptional control, as well as regulation imposed at the level of miRNA processing and action. These additional layers of control provide sophistication and flexibility to the roles played by miRNAs.

Transcriptional Control

A first level of control is transcription. Conserved plant pri-miRNAs usually begin with an adenosine located within 40 nt downstream of a conserved TATA box-like sequence, indicating that ancient plant *MIR* genes are independent transcription units and, thus, have their own promoters (Xie et al., 2005). In addition to biotic or abiotic stress response elements commonly detected in *MIR* promoters (Megraw et al., 2006), tissue-specific or even cell-specific regulatory elements are also likely to exist because conserved *MIR* genes often have highly precise expression patterns (see for example Kawashima et al., 2008; Parizotto et al., 2004; Valoczi et al., 2006). These patterns also agree with the presence of binding sites for known transcription factors involved in developmental patterning. Many conserved *MIR* gene promoters in *Arabidopsis* notably show an overrepresentation of binding sites for the transcription factors ARF (may induce gene repression in the absence of auxin), LFY (activates key floral homeotic genes upon its induction, notably by gibberellic acid, GA), and MYC2 (increases responsiveness to drought through enhanced sensitivity to abscisic acid, ABA). These findings also incidentally uncover a strong link between miRNA transcription and plant hormones (Megraw et al., 2006). Moreover, some of these transcription factor families are themselves regulated by miRNAs, pointing to the existence of complex transcription feedback loops, as has been demonstrated for some metazoan *MIR* genes (Megraw et al., 2006). Phylogenetic shadowing, which identifies sequence elements conserved across species, has been successfully used to identify novel regulatory elements that seem to set apart members of conserved *MIR* multigene families (Warthmann et al., 2008), whose paralogous products are usually considered functionally redundant owing to their identical or near-identical mature sequences. Laser microdissection coupled to RT-PCR showed that, on the contrary, the global pattern of mature *miR-166* accumulation within the maize shoot apex entails exquisite spatio-temporal variations in the transcription of nine distinct *MIR-166* paralogs (Nogueira et al., 2009). This high degree of miRNA cell specificity is likely afforded by independent acquisitions of specific regulatory elements in their respective promoters (Figure 4A).

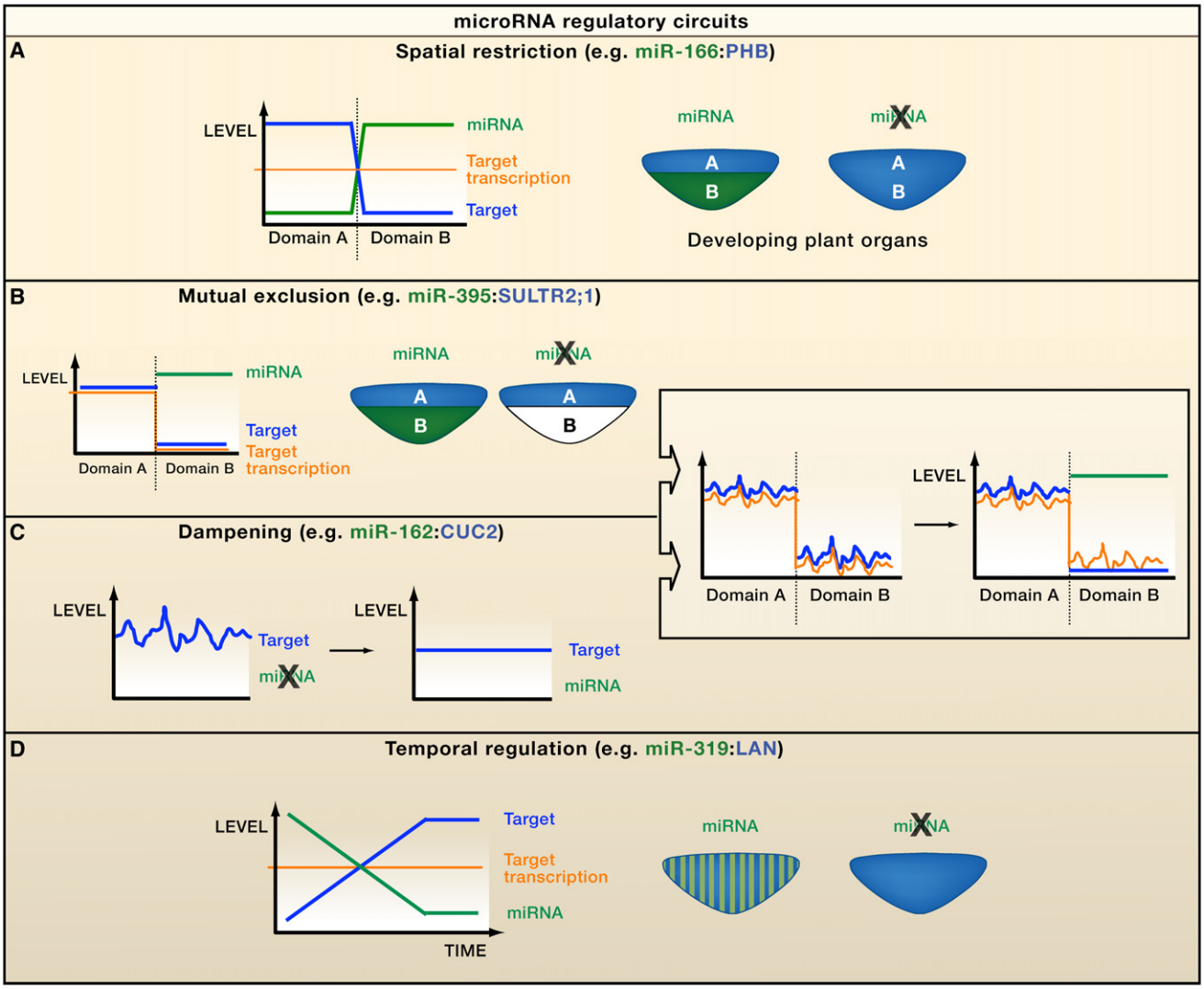

Figure 3. Regulatory Circuits Orchestrated by Plant miRNAs

Consequences of different regulatory circuits on two hypothetical spatially separated domains (A and B) within a developing plant organ. (A) Spatial restriction of target accumulation. Here, the miRNA target accumulates in domain A where the miRNA is not present and the miRNA accumulates in domain B, from which the target is depleted. This mode of regulation is typically observed with HD-ZIP transcription factors (notably *PHB*) regulated by *miR-165/166* paralogs in *Arabidopsis* or maize (Kidner and Martienssen, 2004). (B) In mutual exclusion, the miRNA and its target have spatially separated expression domains. The miRNA acts as a backup to ensure the strict transcriptional confinement of the target within its cognate expression domain. This mode of regulation is possibly illustrated by the *miR-395*-SULTR2;1 interaction in *Arabidopsis* (Kawashima et al., 2008) and also shown in Figure 6A.

(C) Dampening or "thresholding" entails the reduction and stabilization of target gene expression levels in coincidence with miRNA expression, as exemplified by the *miR-164*-CUC2 interaction (Nikovics et al., 2006; Figure 2B). (Box) Dampening and mutual exclusion could possibly also cooperate to further refine the output of miRNA-target interactions.

(D) In temporal regulation, a gradient of miRNA expression generates an opposing gradient of its target over time. The LANCEOLATE (LAN)-*miR-319* interaction in the tomato meristem is an example of this type of regulation (Ori et al., 2007). Note that all these modes of regulation can, in principle, occur by at least two ways of miRNA action (cleavage and/or translational inhibition) according to the scheme presented in Figure 2A.

Promoters for most young *MIR* genes have yet to be characterized, but it can at least be inferred that some proto-*MIR*s formed by inverted duplication initially undergo the same transcriptional regulation as their founder genes (Figure 1B).

Regulating miRNA Processing and Activity

Unlike in animals (Michlewski et al., 2008), there is as yet no experimental evidence supporting miRNA-specific regulations at the processing steps in plants. However, discrepancies between pri-/pre-miRNA and mature miRNA levels frequently observed in northern blot analyses of individual plant miRNAs suggest that these regulations are likely to exist (see for example Nogueira et al., 2009). Several conserved and many nonconserved *Arabidopsis* miRNAs are diced as a 24 nt species, in addition to the canonical and DCL1-dependent 19–21 nt species

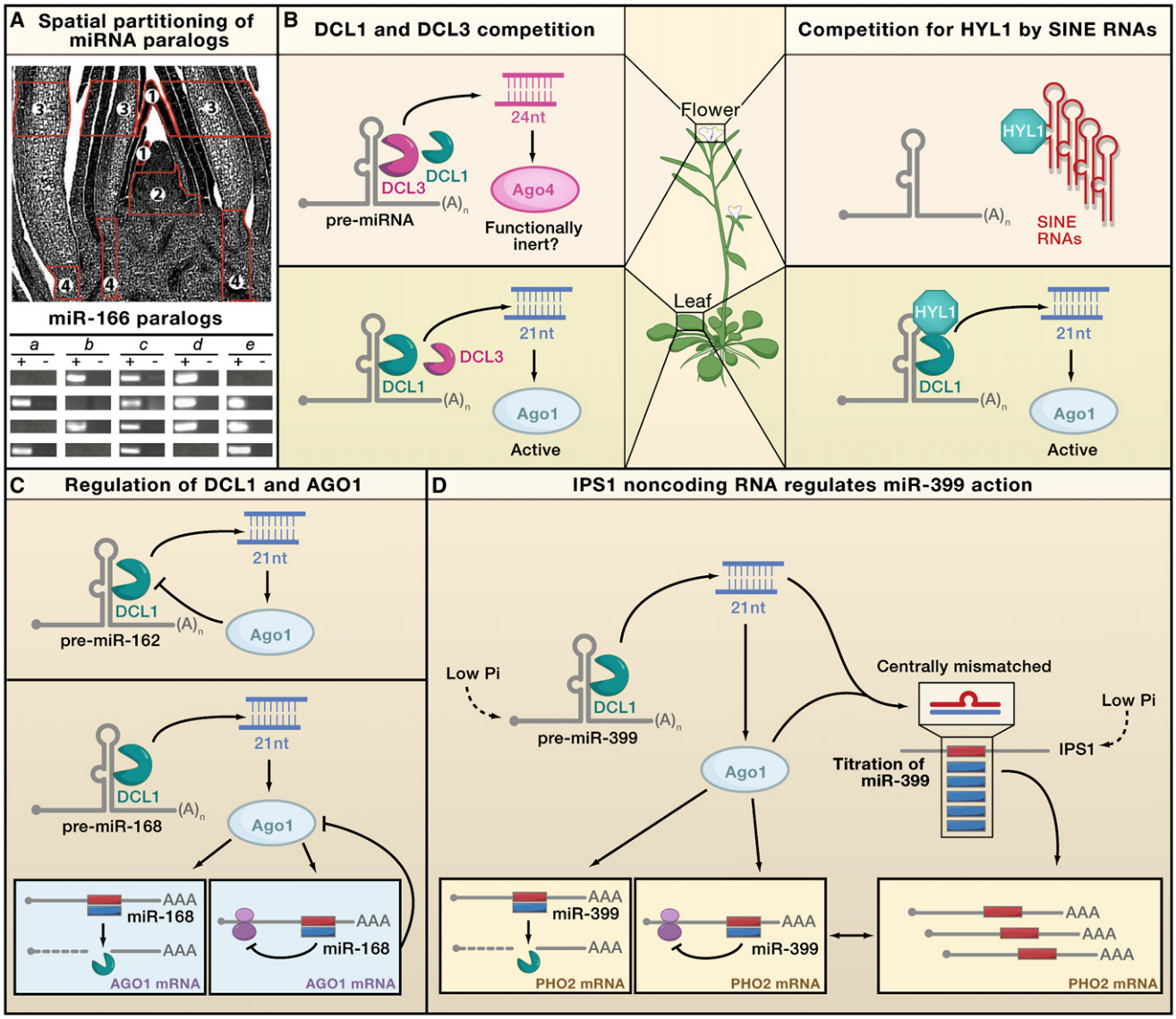

Figure 4. Regulation of Plant miRNA Transcription, Processing, and Activity

(A) *miR-166* paralogs in the maize shoot apical meristem (SAM) are exquisitely spatially partitioned. Laser-assisted microdissection was used to dissect specific domains of the SAM (delineated in red). Quantitative RT-PCR was then used to measure the abundance of *miR-166*-specific isoforms (*miR-166a* to *miR-166e* shown here) in each domain (+, experimental samples; −, negative amplification controls where reverse transcriptase was omitted). Image from Nogueira et al. (2009).

(B) Possible tissue-specific regulations of miRNA processing. (Left) Dicer-like 1 (DCL1), the cognate miRNA processing enzyme, produces 21 nt miRNAs. Leaves expressing DCL1 harbor 21 nt miRNAs. In flowers, DCL3 (the enzyme that normally produces *cis*-acting siRNAs) is more abundant than in leaves. Competition in flowers between DCL1 and DCL3 results in a shift in miRNA size from 21 nt to 24 nt. The possible incorporation of the 24 nt miRNAs into ARGONAUTE 4 (AGO4) instead of AGO1 could result in a loss of posttranscriptional miRNA activity in flowers. (Right) Tissue-specific transcription of short interspaced elements (SINE) RNA could also prevent miRNA production (depicted here as occurring in the flowers). Abundant SINE RNAs that resemble the hairpin structure of miRNA precursors would compete for the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1) that is required for miRNA processing.

(C) Both DCL1 and AGO1 undergo sophisticated homeostatic regulations through the action of *Arabidopsis miR-162* and *miR-168*, respectively, suggesting that miRNA processing and activity can be globally controlled depending on the specific levels of each miRNA.

(D) The IPS1 noncoding RNA provides an efficient means of modulating the activity of *miR-399* and of its target, PHO2, in a sequence-specific manner. Because both *MIR-399* and *PHO2* are induced by a shortage in inorganic phosphate (Pi), this type of regulation could be seen as an example of miRNA (as opposed to target) dampening.

(Dunoyer et al., 2004; Vazquez et al., 2008). This feature is maintained across divergent plant species. It is also apparently manifested in a tissue-specific manner, with the longer miRNA being prevalently produced in the floral structures (inflorescences) where DCL3, the enzyme responsible for its synthesis, is ten times more abundant than in leaves (Vazquez et al., 2008).

Organ-specific competition between DCL1 and DCL3 for miRNA processing might thus constitute a broad regulatory mechanism controlling the production of active miRNA molecules in specific tissues (Figure 4B). Overexpression of short interspaced elements (SINE) RNA in *Arabidopsis* generates phenotypes resembling those of miRNA-deficient mutants. It was found that stem loops of SINE elements mimic the hairpin structures of miRNA precursors and bind to HYL1, the dsRNA-binding protein required for pri-to-pre-miRNA processing (Pouch-Pelissier et al., 2008). These results thus suggest that tissue- or developmental stage-specific expression of SINE RNA might modulate the production of plant miRNA by competing for HYL1 (Figure 4B). They also support the proposal that transposable elements may give rise to at least some plant *MIR* genes (Figure 1D; Piriyapongsa and Jordan, 2008).

Strikingly, *Arabidopsis miR-162* targets DCL1, the main enzyme that processes pre-miRNAs and mature miRNAs in plants (Xie et al., 2003; Figure 1A). Spatial or temporal changes in *miR-162* expression are thus expected to impact the global levels of mature miRNA production in different tissues or at different developmental stages (Figure 4C). The same principles also likely apply to modulation of miRNA activity, as AGO1, the main miRNA effector protein, is itself regulated by *miR-168* in *Arabidopsis* (Vaucheret et al., 2004) (Figure 4C). In addition to miRNA-mediated feedback regulations of the two key enzymes in the pathway, other layers of regulation are likely to exist. For instance, *ago1*, *ago10,* and *vcs* mutations exhibit variable phenotypes in the context of different *Arabidopsis* ecotypes (Goeres et al., 2007; Vaucheret, 2008). This strongly suggests the existence of as yet unidentified modifiers of miRNA effector functions, whose variable expression in space and time could also modulate miRNA-directed silencing.

An unsuspected RNA-based control mechanism over miRNA action was recently discovered following studies of the non-protein-coding gene *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) from *Arabidopsis* (Franco-Zorrilla et al., 2007). The IPS1 RNA contains a motif with sequence complementarity to *miR-399*. Unlike *miR-399* mRNA targets, however, the base-pairing motif in *IPS1* is interrupted by a mismatch loop at the expected miRNA cleavage site, a feature conserved among plant *IPS1* orthologs (Figure 4D). Consequently, the IPS1 RNA is not cleaved following *miR-399* pairing but instead appears to sequester *miR-399*-loaded RISC. Accordingly, engineering IPS1 RNA to permit miRNA cleavage abolishes its *miR-399* inhibitory activity (Franco-Zorrilla et al., 2007). Because *IPS1* transcription is induced like *MIR-399* upon phosphate starvation (Figure 4D), its function may allow the fine-tuning of *miR-399* activity during phosphate shortage (Fujii et al., 2005). Whether this "target mimicry" is used by plants to buffer the effects of other miRNAs remains unclear, but the possibility of a widespread phenomenon certainly deserves attention given the shear abundance of as yet uncharacterized long noncoding RNAs (including antisense RNAs) produced from the genomes of *Arabidopsis* and other plants (Rymarquis et al., 2008). Notably, artificially engineered mimics of target RNAs based on the *IPS1* motif were shown to neutralize the action of cognate miRNAs in transient expression assays (Franco-Zorrilla et al., 2007). This strategy holds great promise for sequence-specific inhibition of plant miRNAs in vivo by using, for example, constitutive or conditionally expressed transgenes (Wang et al., 2008).

Sorting miRNAs into Specific AGO Complexes

One of the great outcomes of deep sequencing is that it permits the exhaustive profiling of AGO-bound small RNAs following immunoprecipitation of AGO protein complexes. This approach was particularly useful in addressing which types of small RNA are predominantly loaded into some of the ten AGO paralogs of *Arabidopsis*. Studies from several laboratories concurred in the finding that the 5′ last nucleotide of small RNA guide strands determines (albeit not always) the identities of at least some of the recruited AGO proteins (Mi et al., 2008; Montgomery et al., 2008a; Takeda et al., 2008). For instance, AGO1 preferentially associates with small RNAs with a uridine at the 5′ terminus, which is possessed by most miRNAs in *Arabidopsis*. In contrast, AGO2 (a protein of as yet unknown function) and AGO4 (which mediates heterochromatic silencing) seem to associate preferentially with small RNAs that bear an adenosine at the 5′ terminus. Although the molecular mechanisms underlying this sorting process have yet to be deciphered, it has potentially important implications for miRNA functions. Indeed, a uridine-to-adenosine change at the 5′ end of engineered miRNAs results in an AGO1-to-AGO2 switch in sRNA loading that abolishes their silencing activity (Mi et al., 2008).

Although artificial, the above example may well reflect situations that are encountered in natural contexts, as deep sequencing of sRNAs isolated from whole tissues shows that miRNAs are rarely cloned as molecules of uniform length or sequence (Figure 5). miRNA variants offset by one or two nucleotides at the 3′ or 5′ ends are not uncommonly uncovered (Fahlgren et al., 2007; Rajagopalan et al., 2006; see also http://asrp.cgrb.oregonstate.edu). It is at present unclear if these variants arise from slight errors during DCL1 processing, or if they reflect bona fide differences in miRNA maturation among the discrete cell types that constitute the tissue sample examined. The existence of flower-enriched DCL3-dependent miRNA species, 24 nt in size, certainly supports the latter idea (Vazquez et al., 2008). Whether they occur in single cells or across distinct cell types, 5′ nucleotide variants of any miRNA could be assembled into different AGO complexes (Figure 5). This could potentially have drastically different regulatory outcomes, ranging from rendering the miRNA functionally inert, as illustrated with the AGO1-to-AGO2 switch, to inducing self-inactivation of miRNA expression. Self-inactivation is, for example, expected to occur with the 24 nt variants of miRNAs because they tend to display more nucleotide polymorphisms at their 3′ and 5′ ends in comparison with their smaller DCL1-dependent counterparts (Vazquez et al., 2008): 5′-adenosine variants of 24 nt-long miRNAs would have all the required features to load functionally into the heterochromatin-inducing AGO4 (Mi et al., 2008; Montgomery et al., 2008a; Takeda et al., 2008). Should some of these miRNA molecules be guanine/cytosine (GC) rich, they would have further potential to operate as *cis*-acting siRNAs by promoting de novo cytosine methylation of their precursor sequences, thereby possibly contributing to the dampening of their own production through transcriptional gene silencing (Figure 5).

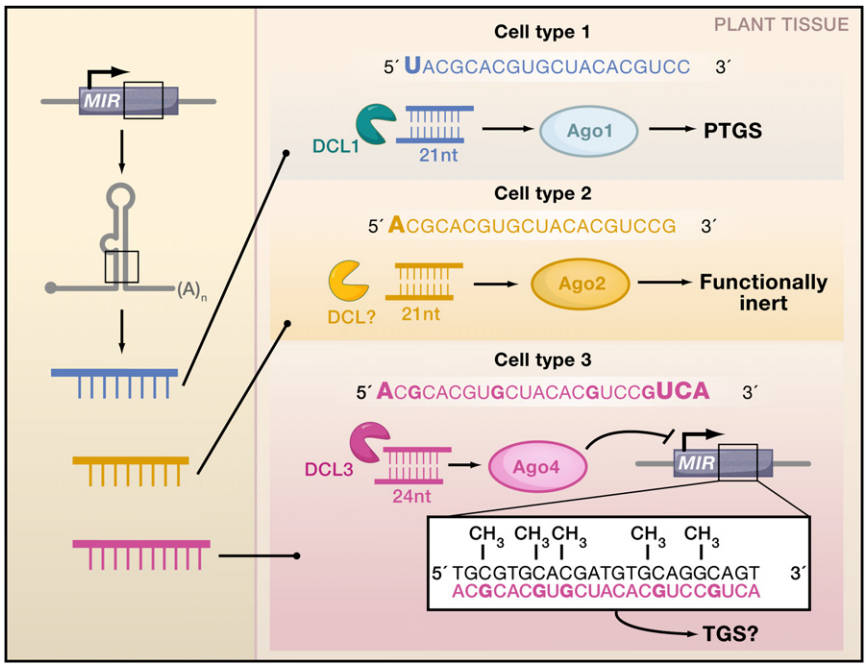

Figure 5. Tissue-Specific miRNA Processing and Sorting

Plant miRNAs are often cloned as discrete molecules of slightly varying length and sequence, represented here by three colors. Although these small differences are usually ascribed to errors occurring during dicing, it is conceivable that such variants reflect bona fide differences in miRNA processing and activities found among the different cell types that constitute the sampled tissue. Here, there are three potentially distinct outputs that depend on the presence or absence of particular Dicer-like (DCL) and ARGONAUTE (AGO) proteins in three cell types. This model takes into account that the length and 5′ terminal nucleotides are important criteria for sorting plant small RNAs into specific AGO complexes. Cognate, 21 nt-long miRNAs with a 5′-uracil (U) are usually the dominant cloned species (blue) and are predicted to be loaded into AGO1 for posttranscriptional gene silencing (PTGS) of expressed target genes in cell type 1. miRNA variants harboring a 5′-adenosine (A) (green), produced specifically in cell type 2, would be loaded into AGO2 and become inert molecules. Longer (24 nt) miRNA variants (purple) with a 5′-adenosine produced by DCL3 rather than DCL1 in cell type 3 would have all the required features to engage with AGO4. This could potentially result in cytosine methylation of the *MIR* gene at the sites of complementarity between the small RNA and *MIR* DNA, possibly resulting in transcriptional gene silencing.

Plant miRNA Action at a Distance?

Whereas the above examples illustrate potential modulation of miRNA processing and action at the single-cell level, controls at the organ or organismal levels are also possible because RNA silencing in plants is not cell autonomous (reviewed in Voinnet, 2005). For instance, the effects of DCL4-dependent siRNA populations derived from RNAi hairpin transgenes or from viruses extend beyond their sites of production (Dunoyer et al., 2007). Movement occurs through the two main routes of macromolecular trafficking in plants: from cell-to-cell through plasmodesmata (the channels connecting all plant cells) and over long distances through the phloem (the tissue that distributes nutrients throughout the plant) (Voinnet, 2005). Nonetheless, although non-cell autonomy is consistent with the immunizing functions of antiviral siRNAs, its possible relevance to miRNA action is debatable. The degree of spatial restriction in *MIR* gene expression (Figure 4A) (Nogueira et al., 2009), the closely correlated patterns of miRNA transcription and activity (Figure 6A) (Parizotto et al., 2004), and the sharply defined regions of mature miRNA localization as detected by in situ hybridization (Valoczi et al., 2006) argue that conserved miRNAs are unlikely to move extensively between plant cells. Even diffuse in situ hybridization signals could not be taken as evidence of miRNA spreading as an initial pool of miRNAs can become progressively diluted and expanded through successive divisions (Figure 6B).

Side-by-side comparisons involving cell-specific expression of an artificial miRNA and of an RNAi hairpin targeting the same endogenous gene (Figure 6C) demonstrated that silencing from the miRNA was restricted to its initiation site, whereas silencing from the RNAi hairpin extended over a range of ten or more cells in the surrounding tissue (Tretter et al., 2008). These observations are particularly intriguing, as the sRNAs involved in these experiments are related in ontology and are both loaded into AGO1 (Dunoyer et al., 2007). The difference in cell autonomy of these two sRNAs suggests that DCL4-dependent siRNA populations (or their precursors), as opposed to DCL1-dependent discrete miRNA molecules, are somehow "qualified" as mobile early during their biogenesis, possibly by specific DCL4-interacting factors. Thus, it is possible that miRNA-mediated silencing might be communicated between cells only in a few instances that involve the action of DCL4. This may well be the case of some inverted-repeat proto-*MIR* genes, which resemble the extended RNAi hairpins used to trigger non-cell-autonomous silencing and are processed by DCL4 (Figures 1B and 6C) (Allen et al., 2004; Rajagopalan et al., 2006; Vazquez et al., 2008).

The production of *trans*-acting siRNAs (tasiRNAs) is an example of a means by which the effects of cell-autonomous miRNAs might be rendered indirectly mobile by the action of DCL4-dependent siRNAs. The biogenesis of tasiRNAs is typically initiated by miRNA-directed cleavage of specific, noncoding precursor transcripts. This promotes complementary strand synthesis that is mediated by RDR6, followed by DCL4-dependent processing of phased 21 nt-long tasiRNAs. These tasiRNAs are then loaded into AGO1 to target in *trans* mRNAs that control processes such as the juvenile-to-adult phase transition and organ polarity (reviewed in Chapman and Carrington, 2007). There are few validated *TAS* loci in *Arabidopsis*, and the cleavage of their precursor transcripts followed by RDR6 action appears to

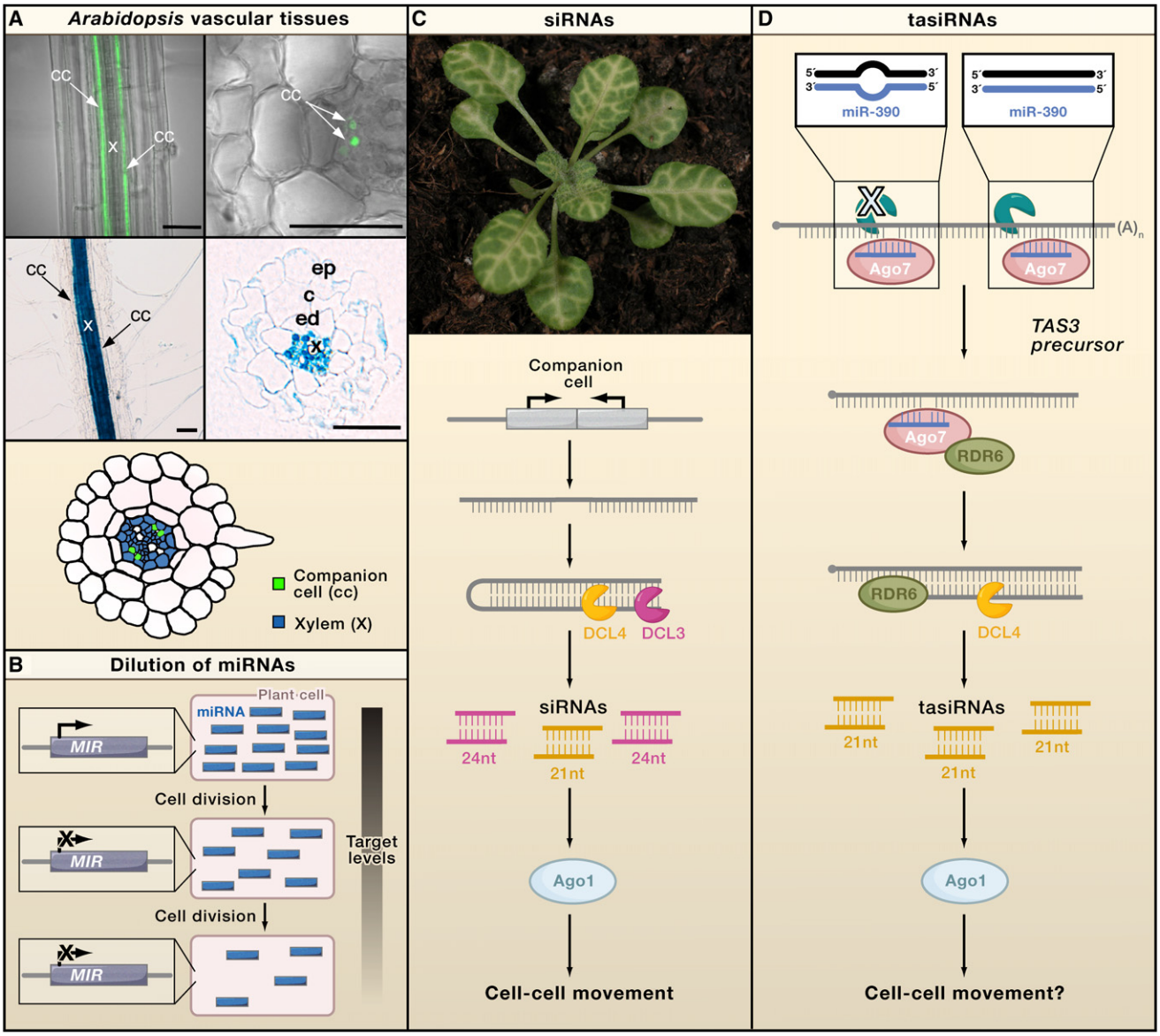

Figure 6. Restricting or Extending miRNA Actions

(A) MicroRNAs can have highly cell-specific patterns of expression or accumulation. (Top) The *miR-395* promoter (green) is only active in phloem companion cells (cc) but not in the xylem (x) of *Arabidopsis* vascular bundles. (Middle) By contrast, its mRNA target, SULTR2;1 (blue), is only expressed in the xylem. (Reprinted with permission from *Plant Journal*, Wiley-Blackwell Publishers, [top] Kawashima et al. [2008] and [middle] Takahashi et al. [2000].) (Bottom) Figure depicts a transverse section of an *Arabidopsis* primary root showing that expression of *miR-395* in the companion cells (green) and of its target SULTR2;1 in xylem parenchyma cells (blue) are mutually exclusive. (Bottom image is courtesy of Hideki Takahashi and Yumiko N. Tsuchiya, RIKEN Plant Science Center, Japan.)

(B) Progressive dilution of an initial pool of miRNAs (blue) through multiple rounds of cell divisions could generate a gradient of target gene expression that could be misinterpreted as evidence of miRNA movement.

(C) RNA silencing mediated by siRNAs can move between cells. (Top) This allows the expansion of silencing into surrounding cells following companion cell (cc)-specific expression of an inverted-repeat RNAi transgene that targets a ubiquitously expressed endogenous gene required for pigmentation. (Bottom) The transgene produces perfectly base-paired double-stranded RNA, which is diced by Dicer-like 4 (DCL4) and DCL3 into siRNA populations 21 nt and 24 nt in length, respectively. The 21 nt siRNAs exert their effects non-cell autonomously through ARGONAUTE 1 (AGO1) (Dunoyer et al., 2007) to produce a chlorotic phenotype in tissues surrounding the vascular system. A highly expressed artificial miRNA targeting the same endogenous gene does not generate any visual phenotype (Tretter et al., 2008) because miRNAs do not spread across cells, thus confining silencing to the companion cells that express the miRNA.

(D) The *trans*-acting siRNA (tasiRNA) pathway (shown here with the *TAS3* locus) may allow miRNAs to indirectly exert effects beyond the cell where they are expressed. The production of tasiRNAs requires two target sites ("double hit"), of which only one site is cleaved (by *miR-390*-loaded AGO7). The resulting DCL4-dependent tasiRNAs resemble 21 nt siRNAs capable of cell-to-cell movement and may thus exert non-cell-autonomous effects on other cells.

rely on unusual features of the transcripts. For example, the TAS3 precursor transcript contains two distinct target sites (double hit) for the highly conserved *miR-390*. One of these sites appears to have evolved in all flowering plants to be in a noncleavable (i.e., centrally mismatched) sequence form, a characteristic that seems to be critical for the production and adequate phasing of tasiRNAs by unknown mechanisms (Axtell et al., 2006).

This double hit configuration of the TAS3 primary transcript, together with the fact that *miR-390* is loaded into AGO7 instead of AGO1, has been suggested to specifically recruit and facilitate RDR6-mediated dsRNA production and subsequent tasiRNA processing by DCL4 (Figure 6D). The TAS1 and TAS2 precursor transcripts show no evidence of a ''double hit,'' yet they too are prone to RDR6 activity upon their cleavage by the *Arabidopsis*-specific *miR-173*. A recent analysis of the *TAS1* locus shows that the *miR-173* target site alone is sufficient to bring reporter transgene transcripts under the control of RDR6 activity (Montgomery et al., 2008b). This is not observed if a *miR-171* target site is engineered, although both *miR-171* and *miR-173* are still loaded into AGO1. Unique and as yet undefined features of the AGO1-*miR-173* complex are thus likely responsible for recruiting RDR6 and DCL4 onto TAS1 transcripts. The sophisticated characteristics of *TAS* loci likely explain why most conventional plant miRNA target transcripts are not prone to generating DCL4-dependent siRNAs upon their cleavage (Voinnet, 2008b) and therefore why they are unlikely to exert their effects between cells. In this respect, tasiRNAs might represent a plant invention to convey the action of a discrete number of miRNAs to cells where they are normally not expressed. This might, for example, generate postdevelopmental gene expression gradients that are relevant to the establishment of polarity in adult plant organs.

Do plant miRNA have the potential to move over long distances in the phloem? Mature miRNA and miRNA* molecules have been cloned from phloem sap of *Brassica napus* plants (Buhtz et al., 2008). Moreover, several miRNAs were detected in the vasculature of *Nicotiana benthamiana* by in situ hybridization (Valoczi et al., 2006). However, these observations may merely reflect the fact that these miRNAs are expressed and exert cell-autonomous functions in nucleated phloem companion cells, as harsh sap extraction procedures (which involve stem severing) might forcibly deliver the miRNAs from the phloem companion cells into the phloem sieve elements, which distributes photo-assimilates (nutrients produced by photosynthesis) throughout the plant. Alternatively, some miRNAs might be translocated by the phloem stream to exert biological functions. Given the wide distribution pattern of photo-assimilates and the highly permissive size exclusion limit of unbranched plasmodesmata found in phloem sink tissues (Oparka et al., 1999), long-distance transport of miRNAs involved in patterning or acquisition of cell identity is unlikely to be advantageous. In contrast, phloem translocation of miRNAs required for stress adaptation may confer benefits to the plant in specifically adapting areas of new growth in response to environmental cues such as local nutrient shortage. In this respect, it is striking that the expression patterns of *MIR-395* and *MIR-399* are both phloem restricted (Aung et al., 2006; Kawashima et al., 2008). Whereas *miR-395* regulates sulfur assimilation genes, *miR-399* targets PHO2/UBC24, a key regulator of phosphate assimilation. Both miRNAs are induced by low nutrients levels (a lack of sulfur and phosphate, respectively) (Chiou et al., 2006; Jones-Rhoades and Bartel, 2004). Upon their induction in leaves, these miRNAs could conceivably be translocated to the roots to increase nutrient uptake and translocation to aerial organs. Consistent with this idea, Pant and colleagues have shown that following micrografting of *miR-399*-overexpressing scions (Pant et al., 2008), transcripts of the *PHO2* gene were targeted by *miR-399* in roots of wild-type rootstocks grown on phosphate-rich medium. Although the exact significance of this phenomenon remains to be established at physiological *miR-399* levels, the results nonetheless suggest that at least some stress-induced miRNAs might on occasion act as systemic silencing signals between distant organs.

Plant miRNA Target Gene Identification

The existence of distinct *MIR* gene classes, of multiple control points in *MIR* expression, and of at least two widespread modes of plant miRNA action all have important implications for miRNA target discovery and validation. So far, near-perfect complementarity has been the exclusive target identification criterion. In addition, 5′ RACE analyses, which unambiguously diagnose endonucleolytic cleavage at the pairing sites between miRNA and presumptive targets, have been the prevalent and successful approaches to experimental validation. Target validation by transcript profiling has also been profitably used (and is still used, particularly in species other than *Arabidopsis*). However, this particular approach can have misleading outcomes because accumulation of the miRNA and of its presumptive target mRNA are usually inferred to be inversely correlated in microarray experiments. This assumes that miRNA and target expression domains coincide in the sampled tissues, which might be true in spatial restriction or dampening modes of regulation (Figures 3A and 3C) but not in mutual exclusion modes of regulation. Mutual exclusion regulatory relationship would not be easily diagnosed by transcript profiling because it entails a simultaneous gain or loss of both miRNA and target expression (Figure 3B). *miR-395* and its target, SULTR2;1, illustrate this notion (Figure 6A). Both miRNA and target are transcriptionally induced upon sulfur starvation because phloem-specific expression of *miR-395* likely functions to prevent leakage in surrounding cell types of xylem-restricted SULTR2;1 expression (Kawashima et al., 2008).

Another approach to plant miRNA target discovery that does not rely on prior knowledge of mature miRNA sequences has recently been implemented. This approach depends on the cloning and sequencing of 3′-cleavage products of miRNA target transcripts (Addo-Quaye et al., 2008; German et al., 2008). Though potentially powerful, this strategy does not take into account the fact that many plant miRNAs might exert their effects mainly by preventing protein production (Figure 2A, left).

The interaction between *miR-834* and its target, COP-INTERACTING PROTEIN 4 (CIP4), provides such an example, where near-complete pairing mediates an on/off type of regulation entirely at the translational level (Brodersen et al., 2008). Thus, extensive miRNA-target pairing does not guarantee even the contribution of slicing to target repression. *miR-834* belongs to a group of young miRNAs assumed to be nonfunctional

because their otherwise near-perfectly complementary targets display unchanged mRNA levels in *dcl1* and *hen1* mutants (Fahlgren et al., 2007; Rajagopalan et al., 2006). Analyzing the CIP4 protein, rather than its mRNA, revealed that *miR-834* is primarily channeled to translational inhibitory pathways. This may also hold true for DCL4-dependent proto-miRNAs (Figure 1B) because even silencing from transgenic RNAi hairpins has a widespread translational inhibitory contribution in *Arabidopsis* (Brodersen et al., 2008). Therefore, in addition to RNA-level analyses, protein profiling is likely necessary for plant target validation and identification, as it already is in metazoan miRNA target studies.

Studies in rice and *Arabidopsis* have uncovered a specific subclass of young miRNAs with no readily identifiable target messages (Fahlgren et al., 2007; Rajagopalan et al., 2006; Wang et al., 2004). A conservative hypothesis holds that those ''orphan'' molecules are from proto-*MIR* genes that have undergone significant sequence drift without selection. However, this idea stems from the use of algorithms specifically designed to identify targets of conserved plant miRNAs, which strongly penalize pairing deviations from perfect complementarity and exclusively scan ORFs (see for instance Rhoades et al., 2002). Yet, from the current genetic data, the existence of extensively mismatched targets repressed mostly at the protein production level, possibly through 5′ or 3′UTR target sites, now warrants serious consideration. Indeed, genes regulated by some orphan miRNAs could fall into this as yet unexplored target category. Analyses of *miR-398* and one of its targets, COPPER SUPEROXIDE DISMUTASE 1 (CSD1), supports and even extends this idea to conserved miRNAs (Dugas and Bartel, 2008). Extensive target mispairs engineered to produce *miR-398*-resistant *CSD1* alleles resulted in compromised slicing, as anticipated. Unexpectedly, however, this modification also rendered the *CSD1* mRNA much more prone to *miR-398*-directed translational repression (Dugas and Bartel, 2008).

How widespread this phenomenon might be is currently unknown, but it raises two concerns regarding the study of plant miRNAs. First, the use of mismatch-tolerating target scan algorithms such as those developed for animal miRNAs might uncover much larger sets of plant targets than currently appreciated. The second concern pertains to the interpretation of ectopic expression experiments involving miRNA-resistant alleles, a common approach to target validation in plants. Only rarely are target protein levels monitored in those experiments. In even fewer cases are the resulting phenotypes compared to those of miRNA knockout mutations (as is done in the experiments depicted in Figure 2C). The *miR-398*-CSD1 precedent, however, suggests that some of the phenotypes generated in those experiments may well be provoked by changes in the miRNA regulatory mode rather than a loss of miRNA function. A shift from irreversible cleavage to reversible translational repression could generate intrinsically unstable developmental cues that would not be induced if the target site is completely removed (the equivalent of which is thought to be achieved by using resistant targets) (Figure 7A). This would possibly cause unsteady phenotypes of variable penetrance, which are indeed observed in some of these experiments (Mallory et al., 2004, 2005).

Another foreseeable problem associated with miRNA-resistant targets is the potential to generate miRNA target mimics (Franco-Zorrilla et al., 2007), particularly if only central mismatches are engineered in overexpressed transgenes to block miRNA action. Here, part of the ensuing phenotype could be due to miRNA titration rather than loss of function (Figure 7B). Even operating as expected, miRNA-resistant target approaches might result in complicated phenotypes difficult to interpret. For example, if several miRNA paralogs are expressed at various levels in distinct tissues, the expression of an miRNA-resistant target in all tissues would engender a full loss-of-function phenotype equivalent to the genetic inactivation of all of the miRNA paralogs (Figure 7C).

Concluding Remarks

Since the first report on plant miRNAs in 2002 (Reinhart et al., 2002), considerable knowledge has been gained in our understanding of their biogenesis, mechanism of action, and possible biological outcomes. Based on the sheer abundance and diversity of plant miRNAs, it is likely that most, if not all, biological processes in plants involve at some point the action of one or more miRNAs. The real impact of most miRNAs in plant biology, however, remains to be ascertained. Until now, few miRNA functions have been studied in their biological context. Many analyses of miRNAs are performed under artificial conditions involving the use of ectopically expressed target transgenes or mutations that confer a gain of function on the miRNA. Only in a few instances has knockout of miRNA-encoding genes been generated and analyzed in *Arabidopsis*. However, it is worth noting that the systematic deletion of 89 miRNA genes in *C. elegans* coupled with in-depth screens for altered morphology, growth, development, and behavior identified only four gene knockouts with detectable phenotypes (Miska et al., 2007). It was concluded from that study that the vast majority of *C. elegans* miRNAs are individually dispensable for major aspects of the nematode's life. These results strikingly mirror the observation that most developmental abnormalities of miRNA-deficient *se-3* mutants can be rescued by mutations in only two targets of *miR-165/miR-166*, *PHABULOSA* and *PHAVOLUTA*, which encode HD-ZIPII transcription factors that specify adaxial cell fates (Grigg et al., 2005). Thus, the establishment of key developmental fates might entail the action of only a small subset of miRNAs in *Arabidopsis* and possibly in other plants.

The many other miRNAs may mainly confer robustness on miRNA-independent transcriptional repression programs. A function for miRNAs as safeguards against unwanted gene expression is a common theme in metazoans (Flynt and Lai, 2008), but this concept has not yet received as much attention in plants. This is possibly due to the fact that most miRNA studies in *Arabidopsis* are conducted under laboratory growth conditions, where the stresses required to induce unsettled transcriptional patterns are usually nonexistent. It could even be argued that the use of such ideal conditions in the laboratory has contributed to selecting an ''elite'' set of plant miRNAs that although used extensively in mechanistic studies may only reflect a part of the biological reality. Thus, studies of miRNAs in crop species that undergo major environmental stresses certainly hold great promise for the identification of new miRNAs and possibly new modes of regulation by these molecules.

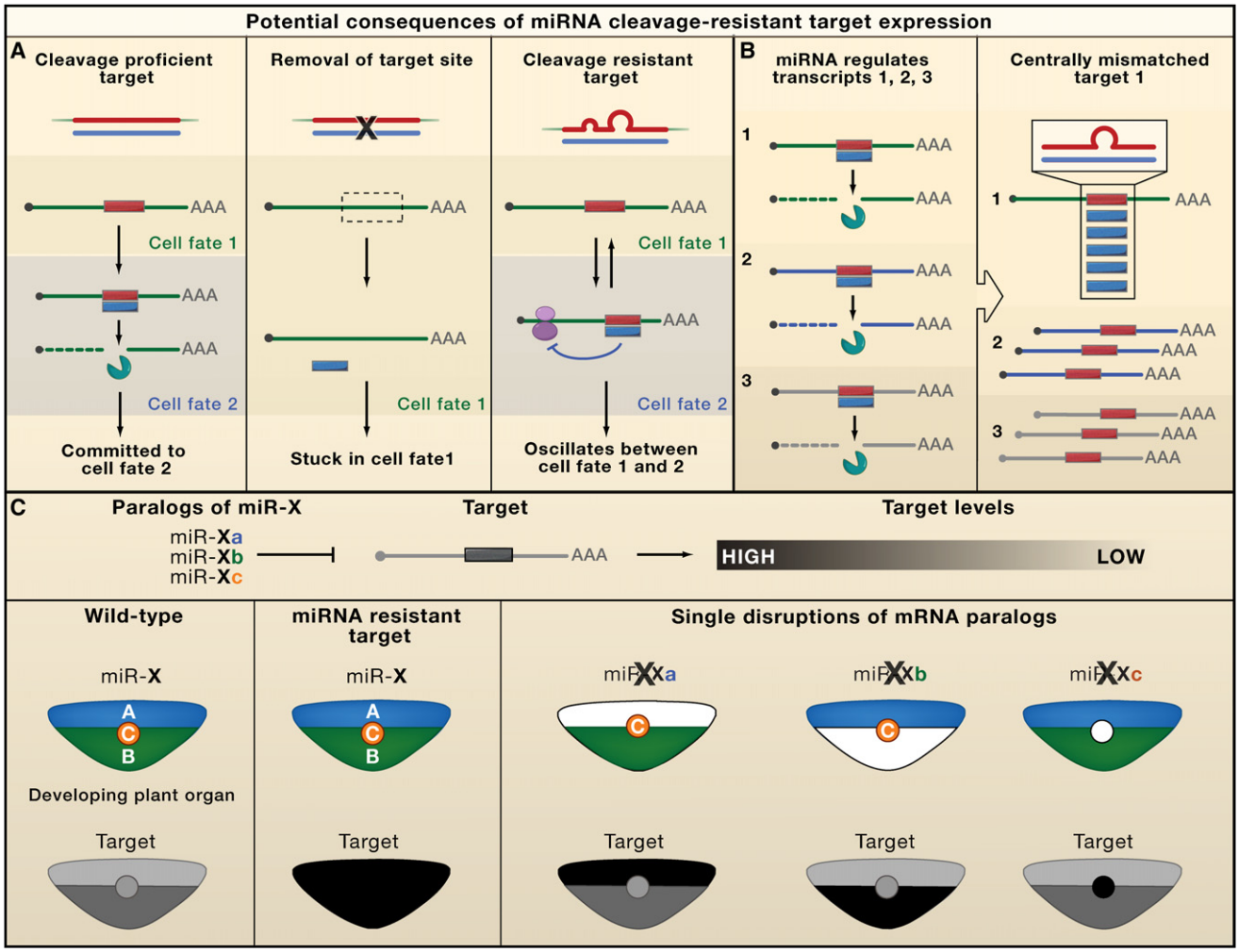

Figure 7. Potential Caveats for Using miRNA-Resistant Target Transgenes in Plant Studies

(A) (Left) The irreversible slicing or "clearance" of a hypothetical important transcription factor transcript specifying cell fate 1 enables the acquisition of cell fate 2 upon the production of the miRNA. (Middle) Complete removal of the miRNA target site permanently locks the cell in fate 1. (Right) Engineering an miRNA-resistant site in the target transcript through mismatches only changes the mode of operation of the miRNA from slicing to reversible translational repression. The cell consequently oscillates between two fates, generating an intrinsically unstable phenotype.

(B) A hypothetical miRNA regulates three distinct types of transcripts (1, 2, 3). If an miRNA-resistant target of transcript 1 is engineered with a central mismatch such that it now behaves as a target mimic by titrating out the miRNA, transcripts 2 and 3 would be freed of miRNA-directed repression as a consequence. These transcripts would accumulate ectopically in the cell, contributing toward an unanticipated exaggerated or distorted phenotype.

(C) (Top) A hypothetical miRNA with three distinct paralogs, each expressed at different levels in specific domains a, b, or c (as in the real example in Figure 4A). (Left) This would normally allow a specific spatial pattern of accumulation of an otherwise ubiquitously expressed target transcript. (Middle) However, transgenic plants producing an miRNA-resistant target would display a strong ectopic target expression phenotype. (Right) This phenotype would not be the same as those generated from disrupting individual miRNA paralogs. Note that a combination of the situations depicted in (A), (B), and (C) would further confuse the interpretation of experiments using miRNA-resistant targets.

The advent of small RNA deep-sequencing technologies has both allowed an unprecedented level of small RNA profiling and uncovered the limits of our capacity to mechanistically or functionally categorize these molecules in plants. These limitations highlight the progressive realization that the schemes of partitioned plant RNA-silencing pathways established through genetic analyses are too reductionist. Indeed, sRNA regulatory modules can be mixed and matched to create a staggering array of regulatory networks in plants with multiple networks possibly operating simultaneously. The discovery of a large and diverse class of young *MIR* genes in plants shows, for example, that the distinction made between siRNA and miRNA can be hazy. In fact, of the many criteria originally used as guidelines for miRNA annotation (Ambros et al., 2003), only one remains strongly reliable in plants: that miRNAs are precisely excised as discrete species from the stem of an imperfect stem-loop precursor, whereas siRNAs occur as populations produced from perfect or near-perfect RNA duplexes (Meyers et al., 2008). A model of

"mRNA clearance" was initially proposed for plant miRNA action in the early days when most identified targets with near-perfect complementarity were found to encode transcription factors involved in key developmental processes (Rhoades et al., 2002). Though this model still holds true (but possibly for a small subset of miRNAs only), it is now obvious that it integrates into a much broader framework of possible regulatory outputs (Figures 2 and 3). Moreover, the currently available data indicate that plant and animal miRNAs are much more similar not only in terms of their activity but also in terms of their biogenesis and genomic evolution, as illustrated throughout this Review. The high degree of conservation between metazoan and plant factors involved in the processing and action of miRNAs suggests that plant and animal miRNAs may not have evolved independently. In fact, as in metazoans, some plant miRNAs may have originated for primordial genome defense purposes from transposable elements through mechanisms that possibly predate the existence of miRNA regulatory networks (Piriyapongsa and Jordan, 2008).

Perhaps the most important aspect emerging from the many studies discussed in this Review is the illustration of how poor our current appreciation is of the spatial and temporal action of plant miRNAs. Although more than 5 million *Arabidopsis* small RNAs have now been sequenced and annotated, accurate expression data for many basic miRNA processor and effector proteins remain unavailable. It is now evident that the output of miRNA action will depend ultimately on the availability of particular miRNA isoforms, specific AGO paralogs, and distinctive mRNA targets within a given cell type (Figure 5). Studies of the *C. elegans lsy-6* miRNA, expressed in less than ten sensory neurons but critical for worm survival (Johnston and Hobert, 2003), certainly prompt the following question: How many important plant miRNAs have so far eluded characterization because of their discrete expression patterns and our current inability to access these patterns?

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